and/or a β-glucanase.

16. A process for the preparation of an animal feed, or a premix or precursor to an animal feed, the process comprising mixing a granulate as defined in any of claims 9 to 15 with one or more animal feed substance(s) or ingredient(s).

17. A process according to claim 16 wherein the mixture of feed substance(s) and granulate is sterilised or treated with steam, pelletised and optionally dried.

18. A composition comprising a granulate as defined in any of claims 9 to 15.

19. A composition according to claim 18 which is an edible feed composition.

20. A composition according to claim 18 or 19 which is an animal feed.

21. A composition according to any of claims 18 to 20 which comprises pellets of one or more feed substance(s) or ingredient(s) mixed with a granulate according to any of claims 9 to 15.

22. A composition according to claim 21 wherein the ratio of granulate:feed substance(s) or ingredients is at least 1g:1kilo (such as at least 100ppm).

23. A composition according to any of claims 18 to 22 which is an animal feed, or a premix or precursor to an animal feed, preparable by a process according to claim 16 or 17.

24. A process for promoting the growth of an animal, the process comprising feeding an animal with a diet that comprises either a granulate as defined in any of claims

9 to 15 or a composition as defined in any of claims 18 to 23.

25. Use of a granulate as defined in any of claims 9 to 15 in, or as a component of, an animal feed or for use in an animal diet.

26. Use of a composition comprising at least 15% (w/w) of an edible carbohydrate polymer as a carrier for an enzyme to improve the pelleting stability of the enzyme.

27. A process for the preparation of an enzyme-containing granulate, wherein an enzyme-containing liquid and a solid carrier comprising at least 15% (w/w) starch are mechanically processed to obtain enzyme-containing granules which are subsequently dried.

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(57) Abstract

A particulate, enzyme-containing preparation suitable for use, e.g., in the manufacture of an animal feed composition comprises: a total of at least 1 % by weight (w/w) of one or more hydrophobic substances; and a total of at least 75 % (w/w) of one or more water-insoluble substances, including the hydrophobic substance(s).

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FEED ENZYME PREPARATIONS

FIELD OF THE INVENTION

The present invention relates to particulate, enzyme-containing preparations [such as a preparation in granulated form (in the following often simply referred to as a "granulate")] which, when the enzyme(s) in question is/are relevant as an ingredient or ingredients of animal fodder, are particularly well suited for incorporation in animal feed compositions, especially animal feed compositions which are formulated as pellets ("pelletized") or the like. When using manufacturing conditions often preferred in the industry for compositions of this type, such as steam-pelletizing, the invention makes possible the manufacture of compositions with a very high degree of retention of enzymatic activity.

BACKGROUND OF THE INVENTION

It is well established that the incorporation of certain types of enzymes, such as β-glucanases, xylanases or phytases, in animal feed (fodder) compositions can have a significant beneficial effect with respect to improvement of nutrient or mineral uptake from the fodder by the animal in question. In the fodder-manufacturing industry, formulation of animal feed compositions in the form of pellets is preferred for a variety of reasons, including: ease of handling and dosing; low levels of dust formation during handling; and ease of digestibility by the animal.

Another - and important - reason why pellet formulations are preferred is that the pelletizing process itself facilitates heat-treatment of the feed for the purpose, for example, of eradicating pathogenic microorganisms, such as *Salmonella* sp., *Listeria* sp., *Campylobacter* sp. and the like; thus, for example, in order to effectively eradicate *Salmonella* sp. the feed must be heat-treated at a temperature of at least about 81°C during

the pelletizing process. This heat treatment is very suitably accomplished by means of, e.g., steam treatment of the feed during the pelletizing process.

Whilst heat treatment of the feed is important for the above-mentioned reason, it is clear that such treatments - particularly treatments involving the use of steam, where the feed ingredients are exposed to both heat and moisture - will generally be expected to have a deleterious (deactivating or denaturing) effect on enzymes present in the composition which is to be treated. The following documents may, by way of example, be mentioned in this connection:

(i) Novo Nordisk publication A-06293 (available from Novo Nordisk A/S, Bagsvaerd, Denmark), which gives enzyme stability data for pelletizing of compositions in which the enzymes are in the form of coated granulates containing xylanase and β-glucanase from *Humicola insolens*, amylase from *Bacillus subtilis*, and protease from *Bacillus licheniformis*, respectively. Steam-heating to 85°C is reported to result in a loss of of ca. 25% of the initial enzyme activity in each case.

(ii) Novo Nordisk Product Sheet No. B402e-GB (available from Novo Nordisk A/S, Bagsvaerd, Denmark), which reports that a coated enzyme granulate known as Bio-Feed™ Plus CT [containing a carbohydrase preparation (Bio-Feed™ Plus) produced by submerged fermentation of *Humicola insolens*] will retain more than 75% of its activity in a feed-pelletizing process where the feed ingredients (comprising the enzyme granulate) are heated to 83°C.

(iii) EP 0 569 468 B1, which discloses (see Example 1 therein) that a coated form of a granulated enzyme preparation designated therein as "Bio-Feed Plus T" [the latter being a granulate of the so-called "T-granulate" type, i.e. a granulate produced in accordance with US 4,106,991 and which in addition to enzyme(s)

comprises, *inter alia*, 2-40% w/w of finely divided cellulose fibers] retained 90-100% of its original fungal β-glucanase activity when incorporated as an ingredient in a fodder composition subjected to a pelletizing procedure lasting 25-30 seconds and involving a temperature of 70°C and direct steam injection. In contrast, uncoated T-granulate as well as a coated, "Bio-Feed Plus" granulate which was not of the T-granulate type both exhibited lower (75%) retention of fungal β-glucanase activity.

It is well known that different enzymes can have widely differing properties, notably with respect to their thermal stability, e.g. their ability to withstand relatively high temperatures. Moreover, it is the present inventors' experience that enzyme-containing granulates of the coated T-granulate type (*vide supra*) can display widely differing stability (notably thermal stability, e.g. stability during pelletizing processes), depending on the nature and amounts of the non-enzyme constituents of the granulate, and the same applies in the case of enzyme-containing granulates of types other than the coated T-granulate type.

An object of the investigations underlying the present invention was therefore to achieve increased stability of enzyme preparations in particulate form, e.g. enzyme-containing granulates, under conditions of heat and moisture such as those - briefly outlined above - obtaining in processes (e.g. pelletizing processes) employed in the animal feed manufacturing industry.

DESCRIPTION OF THE INVENTION

The present inventors have surprisingly found that significantly enhanced retention of enzymatic activity of particulate, enzyme-containing preparations (e.g. granulates) under pelletizing conditions as mentioned above can be achieved by inclusion in the particulate, enzyme-containing formulation of appropriate

proportions of (i) a hydrophobic material, particularly when some or all of the hydrophobic material (which may, by way of example, be a waxy material, or a fatty material such as tallow) is present in a coating layer encapsulating an enzyme-containing core of the particles, and (ii) water-insoluble material (including the hydrophobic material mentioned above).

Thus, a first aspect of the present invention relates to a particulate, enzyme-containing preparation (e.g. a granulate) comprising: a total of at least 1% by weight (w/w) of one or more hydrophobic substances; and a total of at least 75% w/w of one or more water-insoluble substances, including the hydrophobic substance(s).

It is preferred that the hydrophobic substance(s) constitute at least 5% w/w of the enzyme-containing preparation, more preferably often at least about 8% w/w. The water-insoluble substance(s) preferably constitute at least 80% w/w, more preferably at least 85% w/w, for example at least 90% w/w of the enzyme-containing preparation.

The weight percentages referred to are based on the total weight of the finished preparation (i.e. including, e.g., any coating layer which may be present).

In uncoated preparations according to the invention it is generally preferable that the hydrophobic substance(s) is/are distributed substantially evenly throughout the material of the particles of the preparation (or at least throughout the material of typical particles taken from the preparation). The content of hydrophobic substance(s) in such preparations may suitably be in the range of 5-95% w/w. In certain cases, a content of hydrophobic substance(s) as high as about 98% w/w may even be appropriate for a particulate, enzyme-containing preparation of this type.

As already indicated to some extent above, preferred

particulate, enzyme-containing preparations of the invention comprise (a) an enzyme-containing core and (b) a coating layer which contains the hydrophobic substance(s) and which surrounds or encapsulates the enzyme-containing core. In such preparations, the hydrophobic substance(s) will suitably constitute from 1 to 50% w/w of the preparation, often from 5 to 25% w/w, and more typically from 5 to 15% w/w thereof.

The term "particulate" as employed in the context of enzyme-containing preparations according to the present invention is intended to embrace particles of any shape or form, such as particles having some essentially regular (e.g. spherical) geometry, particles of irregular shape, or particles in the form of regular or irregular flakes, as well as mixtures of particles having different shapes or forms (such as mixtures of the latter-mentioned types of particles).

Enzymes

Enzyme classification numbers (EC numbers) referred to in the present specification with claims are in accordance with the Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press Inc., 1992.

The enzyme(s) in a particulate, enzyme-containing preparation according to the invention may be any type of enzyme, in particular any type of enzyme of relevance in the context of animal feed compositions. Thus, for example, the enzyme(s) may be chosen among phytases [in particular 3-phytases (i.e. *myo*-inositol-hexakisphosphate 3-phosphohydrolases; classified under EC 3.1.3.8) or 6-phytases (i.e. *myo*-inositol-hexakisphosphate 6-phosphohydrolases; classified under EC 3.1.3.26)], phosphatases (e.g. enzymes classified under EC 3.1.3.1 or EC 3.1.3.2), xylanases (e.g. enzymes classified under EC 3.2.1.8 or EC 3.2.1.32), β-glucanases (e.g. enzymes classified under EC 3.2.1.75, EC 3.2.1.71, EC 3.2.1.59 or EC 3.2.1.39), β-

galactanases [e.g. enzymes classified under EC 3.2.1.89 (endo-1,4-β-galactanases) or EC 3.2.1.90 (endo-1,3-β-galactanases)], α-galactosidases (e.g. enzymes classified under EC 3.2.1.22), β-galactosidases [e.g. enzymes classified under EC 3.2.1.23 ("lactases")], α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), cellulases (e.g. enzymes classified under EC 3.2.1.4), pectinases (e.g. enzymes classified under EC 3.2.1.15) and peptidases (EC 3.4), including "proteinases" or "proteases", such as enzymes classified under EC 3.4.21.

As will already be clear from the above, one or more different enzymes may be incorporated in a particulate, enzyme-containing preparation according to the invention. The manner in which the enzyme content of the particles of an enzyme-containing preparation of the invention is distributed within the particles appears to be unimportant. The enzyme content may be thus be distributed evenly or unevenly throughout the material of a given particle.

In the case of coated particles, the enzyme content of the particles may be substantially confined to the core of the particles, or it is recognized that at least some of the enzyme content may be present in the coating layer(s).

The content of enzyme protein in a particulate, enzyme-containing preparation of the invention will generally be in the range of 0.01-10% by weight, such as 0.1-10% by weight (w/w), of the preparation, more typically 0.2-5% w/w, often 0.3-2.5% w/w. The enzyme content will depend to a large extent of the nature of the enzyme(s) present in the particulate preparation. The particulate preparation may also contain other material originating from the enzyme production process, in that it is preferred practice in the industry to employ enzyme preparations which are not completely purified for incorporation in particulate preparations in general.

Particles

The particulate, enzyme-containing preparation of the invention may, as already indicated to some extent (*vide supra*), very suitably be a granulate (i.e. in granulated form), such as a "T-granulate" (*vide supra*) or some other type of granulate. Alternatively, the particles of a particulate, enzyme-containing preparation of the invention may, for example, be particles comprising a suitable proportion of a meltable component - such as palm oil (and/or another meltable vegetable oil or fat), hydrogenated palm oil (and/or another hydrogenated vegetable oil), tallow, hydrogenated tallow or a wax - which functions as a matrix for the enzyme(s) and other ingredients. In the manufacture of particles of this type, the enzyme(s) and other relevant ingredients of the preparation are introduced into the melted, meltable component, and the melt is then allowed to solidy under particle-forming conditions.

With respect to the size of the particles in a particulate, enzyme-containing preparation of the invention, from the point of view of facilitating adequate mixing with the other ingredients of the feed and ensuring adequate evenness of distribution of enzyme(s) in the final feed product, a practical upper limit for the particle size of the majority of particles in a preparation to be incorporated in a feed composition will normally be about 2 mm.

In this context, the size of a particle may be regarded as the greatest linear dimension of the particle; thus, in the case of, e.g., a substantially spherical particle (such as a substantially spherical granulate particle), the particle size in question will be the diameter of the particle.

The lower limit to the size of particles of an enzyme-containing preparation of the invention will normally be set primarily by the desirability of avoiding dust formation during production and handling of the preparation. A practical lower limit for the

size of such particles will often be about 0.1 mm.

Hydrophobic substances

The term "hydrophobic substance" as employed in the context of the present invention designates a substance which is not readily wetted by water, i.e. which tends to repel water. Such substances - examples of which are oils, fats, hydrocarbon waxes and numerous types of resins - are in general essentially completely insoluble in water.

Hydrophobic substances which are of particular relevance in the context of the present invention are normally substances which are soluble in organic solvents of the hydrocarbon type (e.g. hexane, heptane and the like) or chlorinated hydrocarbon type (e.g. dichloromethane, chloroform and the like). Suitable examples hereof include various glyceride lipids (i.e. mono-, di- or triglycerides), such as animal tallow (e.g. beef or mutton tallow) and vegetable oils, and certain derivatives thereof.

Particularly well suited hydrophobic substances are those which are solid at ambient temperature and which have a melting point of about 40°C or above. Examples hereof include substances such as certain native or hardened (hydrogenated) vegetable oils or fats, e.g. hydrogenated palm oil, hydrogenated palm kernel oil or hydrogenated soya bean oil, as well as materials such as hydrogenated tallow (e.g. hydrogenated beef tallow or mutton tallow).

An assay procedure which is generally suitable for determination of the total content of hydrophobic substances (as defined herein) in a particulate, enzyme-containing preparation according to the invention, and which makes use of extraction with dichloromethane and n-hexane, is described in the Materials and Methods section herein (*vide infra*).

Water-insoluble substances

The term "water-insoluble substance" as employed in the context of the present invention embraces substances ranging from those which are essentially completely insoluble in water to those which exhibit a very low degree of solubility in water (such as a solubility of, say, at most about 1 gram per 100 ml of pure water). Thus, water-insoluble substances in the context of the invention include, but are not limited to, hydrophobic substances as defined above. Other types of water-insoluble substances of relevance in the context of the invention include various inorganic salts (e.g. calcium carbonate, calcium sulfate, calcium hydrogen phosphate or magnesium carbonate), mineral materials comprising aluminium silicates (e.g. clays, such as kaolin, bentonite or fuller's earth), inert metal oxides (e.g. titanium dioxide or magnesium oxide), various macromolecular substances [e.g. polysaccharides, such as cellulose or certain types of starch, as well as milled cereals or cereal flour (e.g. produced from cereals such as wheat, barley or rye), or soya (e.g. soya meal or soya flour)] and activated carbon.

An assay procedure which is generally suitable for determination of the total content of water-insoluble substances (as defined herein) in a particulate, enzyme-containing preparation according to the invention is described in the Materials and Methods section herein (*vide infra*).

Further aspects of the invention

The present invention further relates to a process for the production of an enzyme-containing animal feed composition, wherein an enzyme-containing preparation according to the invention, in admixture with other constituents of the final feed composition, is subjected to a steam-heating step (such as is often involved in pelletizing procedures as outlined above, in which the final feed composition is pelletized).

A further aspect of the invention relates to the use of a particulate, enzyme-containing preparation according to the invention in the manufacture of an enzyme-containing animal feed composition.

In this connection, the present invention also encompasses:

an enzyme-containing feed composition manufactured using, as enzyme-containing starting material, a particulate, enzyme-containing preparation according to the invention; and to

an enzyme-containing animal feed composition obtained or obtainable by a production process (outlined above) according to the invention.

It will be apparent that enzyme-containing, particulate preparations formulated in accordance with the invention may also be employed in the production of other (non-feed) enzyme-containing compositions whose manufacture entails a heat-treatment as outlined herein.

It is further envisaged that the principles of the present invention are applicable not only to improving the retention of enzyme activity in connection with heat-treatment (e.g. steam-treatment) of enzyme-containing preparations, but may be employed analogously to achieve, for example, improved stability of vitamins, co-vitamins, amino acids, medicaments (e.g. antibiotics or growth-regulating substances), mineral additives and the like, or enhanced viability of microorganisms (e.g. bacteria or fungi), following heat-treatment (e.g. steam-treatment) of particulate preparations incorporating these.

The invention is further illustrated, but in no way limited, by the examples given in the Materials and Methods section, below.

MATERIALS AND METHODS

General description of preparation of T-granulates

As mentioned earlier, granulates of the "T-granulate" type are characterized in US 4,106,991.

The coated T-granulates described in Examples 1 and 2, below (all of which are examples of granulates wherein the requisite hydrophobic substance is incorporated in the coating), were prepared by the general procedure described hereinafter, employing the following starting materials:

1) cellulose fibres (Arbocel™ BC 200)

2) finely ground "filler" (which in the case of preparations according to the invention comprises one or more water-insoluble substances within the context of the invention)

3) dextrin (TACKIDEX™ GM 155 or AVIDEX™ 28LA21) and/or another carbohydrate-type binder, and

4) aqueous enzyme solution.

[reference is made to Examples 1 and 2 (*vide infra*) for further details].

Granulation was carried out in a Lödige mixer, equipped with the necessary mixing paddle and rotating knives.

After thoroughly mixing the cellulose fibres with the filler and part of the binder, an aqueous solution containing the enzyme and the remaining part of the binder was sprayed into the mixer, resulting in the formation of granulate particles through the action of the rotating knives.

The granulate was then transferred to a fluid-bed dryer for drying, and the dried granulate was then cooled and sieved.

Coating of the resulting T-granulate particles with a coating comprising hydrophobic substance(s) was also carried out in a Lödige mixer:

The dry granulate particles were heated to a temperature in the

range of about 70-85°C, usually 75-85°C (depending on the melting characteristics of the hydrophobic component employed). The hydrophobic component was melted and sprayed evenly onto the granulate particles in the mixer. A second filler was incorporated in the manner described in EP 0 569 468 B1. After application of the hydrophobic component and filler, the resulting T-granulate was cooled to ambient temperature and sieved to remove very fine particles and undesirably large particles.

All the T-granulates employed in the working examples given hereafter had a predominant particle size (diameter) in the range of 0.3-1.2 mm.

Assay for determining the total content of hydrophobic substances in a preparation of the invention

Approximately 10 grams of the preparation in question (exact weight W grams) are agitated intermittently in 50 ml of dichloromethane in a tightly closed vessel for about 4 hours at 20-25°C. A 50 ml aliquot of n-hexane is added, and the vessel is then re-closed, agitated and allowed to stand for several hours until the upper part of the liquid phase appears clear. A 10 ml sample of the clear liquid phase is transferred to a 50 ml glass beaker of known weight (to 4 decimals), and the solvent is allowed to evaporate completely. The beaker is then re-weighed (again to 4 decimals) to determine the weight (R, in grams) of the residue remaining after evaporation.

The percentage total content of hydrophobic substances is then calculated as

$[(R \times 10)/W] \times 100$.

Assay for determining the total content of water-insoluble substances in a preparation of the invention

Approximately 10 grams of the preparation in question (exact weight W grams) are stirred in 50 ml of de-ionized water in a closed vessel for 2 hours at 20-25°C. A sample of the resulting

suspension/slurry is centrifuged, and the percentage dry solids content, S, of the clear supernatant is determined refractometrically, employing the °Brix scale, to the nearest 0.1%.

The percentage total content of water-insoluble substances is then calculated as:
100 - [(S x 50)/W].

EXAMPLE 1

Preparation of phytase-containing, granulate preparations: Five different phytase-containing granulates were prepared by the T-granulation procedure (*vide supra*). The phytase solution used in the granulations was from the same source as that used in the production of the product Phytase Novo L (see Novo Nordisk Product Sheet B-722a, available on request from Novo Nordisk A/S, Bagsvaerd, Denmark). Phytase Novo L is a liquid enzyme formulation with a standardised phytase activity.

The five granulates, designated PA, PB, PC, PD and PE, respectively, were prepared using the following constituents in the amounts specified (given on a dry matter basis):

Granulate PA (coated)

Constituent	% w/w of preparation	
	Core	Coating
Sodium chloride	54	
Kaolin	3	9
Cellulose fibres	9	
Calcium carbonate		9
Dextrin + sucrose + enzyme	8	
Hydrogenated beef tallow		9

As is apparent from the above table, the main filler employed in granulate PA was the water-soluble salt sodium chloride, and the total contents of hydrophobic substance (hydrogenated beef tallow) and water-insoluble substances (hydrogenated beef tallow + kaolin + cellulose + calcium carbonate) were 9% w/w and 39% w/w, respectively. The latter values agree well with the values (8% w/w and 37%, respectively) determined employing the assay procedures described above. The phytase activity of granulate PA was determined as 2400 FYT/g, using Novo Nordisk analytical method KAL-SM-0403.01/01 (available on request from Novo Nordisk A/S, Bagsvaerd, Denmark).

Granulate PB (uncoated)

Constituent	% w/w of preparation
Cellulose fibres	11
Calcium carbonate	73
Dextrin + enzyme	16

As is apparent from the latter table, the main filler employed in granulate PB was the water-insoluble salt calcium carbonate, and the total content of water-insoluble substances was 84% w/w. This value is in excellent agreement with the value (84% w/w) determined employing the assay procedures described above for water-insoluble substances. No hydrophobic substance (as defined herein) was employed, which is consistent with the finding of <1% w/w of hydrophobic substance using the above-described assay for hydrophobic substances. The phytase activity of granulate PB was determined as 1760 FYT/g, using Novo Nordisk analytical method KAL-SM-0403.01/01 (*vide supra*)

Granulate PC (coated)

Constituent	% w/w of preparation	
	Core	Coating
Kaolin		7
Cellulose fibres	6	
Calcium carbonate	54	7
Dextrin + enzyme	12	
Hydrogenated beef tallow		11

As is apparent from the latter table, the main filler employed in granulate PC was the water-insoluble salt calcium carbonate, and the total contents of hydrophobic substance (hydrogenated beef tallow) and water-insoluble substances (hydrogenated beef tallow + kaolin + cellulose + calcium carbonate) were 11% w/w and 87% w/w, respectively. The latter values agree well with the values (10% w/w and 87%, respectively) determined employing the assay procedures described above. The phytase activity of granulate PC was determined as 950 FYT/g, using Novo Nordisk analytical method KAL-SM-0403.01/01 (*vide supra*).

Granulate PD (coated)

Constituent	% w/w of preparation	
	Core	Coating
Kaolin	6.3	7.8
Cellulose fibres	9.4	
Calcium carbonate		7.8
Calcium hydrogen phosphate	48.5	
Dextrin + enzyme	14.5	
Hydrogenated palm oil		5.5

As is apparent from the latter table, the main filler employed in granulate PD was the water-insoluble salt calcium hydrogen phosphate ($CaHPO_4$), and the total contents of hydrophobic substance (hydrogenated palm oil) and water-insoluble substances (hydrogenated palm oil + kaolin + cellulose + calcium carbonate + calcium hydrogen phosphate) were 5.5% w/w and 79.8% w/w, respectively. The phytase activity of granulate PD was determined as 1430 FYT/g, using Novo Nordisk analytical method KAL-SM-0403.01/01 (*vide supra*).

Granulate PE (coated)

Constituent	% w/w of preparation	
	Core	Coating
Kaolin	5.1	9.3
Cellulose fibres	7.9	
Calcium carbonate		9.3
Sodium sulfate (anhydrous)	42.8	
Dextrin + enzyme	17.1	
Hydrogenated palm oil		8.1

As is apparent from the latter table, the main filler employed in granulate PE was the water-soluble salt sodium sulfate, and the total contents of hydrophobic substance (hydrogenated palm oil) and water-insoluble substances (hydrogenated palm oil + kaolin + cellulose + calcium carbonate) were 8.5% w/w and 40.1% w/w, respectively. The phytase activity of granulate PE was determined as 2400 FYT/g, using Novo Nordisk analytical method KAL-SM-0403.01/01 (*vide supra*).

EXAMPLE 2

Preparation of xylanase-containing, granulate preparations: Five different xylanase-containing granulates were prepared by the T-granulation procedure (*vide supra*). The xylanase solution used in the granulations was from the same source as that used in the production of the product Bio-Feed™ Wheat L (see, e.g., Novo Nordisk publication B-854a, available on request from Novo Nordisk A/S, Bagsvaerd, Denmark). Bio-Feed™ Wheat L is a liquid enzyme formulation with a standardised xylanase activity.

The five granulates, designated XA, XB, XC, XD and XE, respectively, were prepared using the following constituents in

the amounts specified (given on a dry matter basis):

Granulate XA (coated)

Constituent	% w/w of preparation	
	Core	Coating
Sodium sulfate (anhydrous)	55	
Cellulose fibres	7	
Calcium carbonate	3	18
Dextrin + enzyme	10	
Hydrogenated beef tallow		8

As is apparent from the above table, the main filler employed in granulate XA was the water-soluble salt sodium sulfate, and the total contents of hydrophobic substance (hydrogenated beef tallow) and water-insoluble substances (hydrogenated beef tallow + cellulose + calcium carbonate) were 8% w/w and 36% w/w, respectively. The latter values agree well with the values (9% w/w and 36%, respectively) determined employing the assay procedures described above. The xylanase activity of granulate XA was determined as 810 FXU/g, using Novo Nordisk analytical method KAL-SM-0356.01/01 (available on request from Novo Nordisk A/S, Bagsvaerd, Denmark).

Granulate XB (coated)

Constituent	% w/w of preparation	
	Core	Coating
Calcium sulfate	54	
Kaolin	3	
Cellulose fibres	7	
Calcium carbonate		18
Dextrin + enzyme	9	
Hydrogenated beef tallow		8

As is apparent from the latter table, the main filler employed in granulate XB was the water-insoluble salt calcium sulfate, and the total contents of hydrophobic substance (hydrogenated beef tallow) and water-insoluble substances (hydrogenated beef tallow + calcium sulfate + kaolin + cellulose + calcium carbonate) were 8% w/w and 90% w/w, respectively. The latter values are in excellent agreement with the values (8% w/w and 90%, respectively) determined employing the assay procedures described above. The xylanase activity of granulate XB was determined as 770 FXU/g, using Novo Nordisk analytical method KAL-SM-0356.01/01 (*vide supra*).

Granulate XC (uncoated)

Constituent	% w/w of preparation
Sodium sulfate (anhydrous)	76
Cellulose fibres	11
Calcium carbonate	6
Dextrin + enzyme	8

As is apparent from the latter table, the main filler employed in granulate XC was the water-soluble salt sodium sulfate, and the total content of water-insoluble substances was 17% w/w. The value determined employing the assay procedure described above for water-insoluble substances was 23% w/w. No hydrophobic substance (as defined herein) was employed, which is consistent with the finding of <1% w/w of hydrophobic substance using the above-described assay for hydrophobic substances. The xylanase activity of granulate XC was determined as 1080 FXU/g, using Novo Nordisk analytical method KAL-SM-0356.01/01 (*vide supra*).

Granulate XD (uncoated)

Constituent	% w/w of preparation
Calcium sulfate	73
Kaolin	4
Cellulose fibres	10
Dextrin + enzyme	12

As is apparent from the latter table, the main filler employed in granulate XD was the water-insoluble salt calcium sulfate, and the total content of water-insoluble substances was 87% w/w. This agrees well with the value (88% w/w) determined employing the assay procedure described above for water-insoluble substances. No hydrophobic substance (as defined herein) was employed, which is consistent with the finding of <1% w/w of hydrophobic substance using the above-described assay for hydrophobic substances. The xylanase activity of granulate XD was determined as 1090 FXU/g, using Novo Nordisk analytical method KAL-SM-0356.01/01 (*vide supra*).

Granulate XE (coated)

Constituent	% w/w of preparation	
	Core	Coating
Cellulose fibres	8	
Calcium carbonate	54	18
Dextrin + enzyme	12	
Hydrogenated beef tallow		8

As is apparent from the above table, the main filler employed in granulate XE was the water-insoluble salt calcium carbonate, and the total contents of hydrophobic substance (hydrogenated beef tallow) and water-insoluble substances (hydrogenated beef tallow + cellulose + calcium carbonate) were 8% w/w and 88% w/w, respectively. The latter values agree quite well with the values (6% w/w and 90% w/w, respectively) determined employing the assay procedures described above. The xylanase activity of granulate XE was determined as 410 FXU/g, using Novo Nordisk analytical method KAL-SM-0356.01/01 (*vide supra*).

EXAMPLE 3

Effect of steam-heating on phytase activity of phytase-containing feed formulations: The degree of retention of enzymatic activity following steam-heating of feed formulations incorporating one of the phytase-containing granulate preparations PA, PB, PC, PD and PE (see Example 1), respectively, was examined:

A mash feed formulation of the following composition (w/w) was chosen:

62% wheat
15% soya bean meal
12% barley
7% fish meal
2% soya bean oil
1% calcium carbonate
1% dicalcium phosphate
0.2% Solivit™ Mikro 106*

*a commercial vitamin/mineral additive

For each of the phytase-containing granulate preparations PA, PB, PC, PD and PE, the preparation in question was mixed thoroughly into a portion of the above feed formulation. In the case of granulates PA, PB and PC the mixing ratio was such as to give a phytase activity in the final feed formulation in the range of about 1500-2500 FYT/kg, whilst in the case of the granulates PD and PE the mixing ratio was such as to give a phytase activity in the final feed formulation in the range of about 3000-4000 FYT/kg. The phytase activity of a sample of each of the five resulting phytase-enriched feed formulations was determined using Novo Nordisk analytical method ED-9513614, described below. The endogenous phytase activity of a sample of the above-described feed formulation to which no phytase-containing preparation had been added was determined in the same manner, and the value herefor was subtracted from the value for each phytase-enriched feed formulation.

Analytical method

1. Analytical Principle and Unit Definition: In Novo Nordisk analytical method ED 9513614, the phytase is extracted from the feed in an aqueous solution and the enzyme is then allowed to react with *myo*-inositol hexakisphosphate (phytate) under standardised conditions (temperature 37°C, pH 5.5 with acetate buffer, reaction time 60 minutes, initial phytate concentration 5 mM). The reaction is terminated, and inorganic phosphate is

determined by addition of molybdate/vanadate in nitric acid. Blank values are determined by addition of the molybdate/vanadate/nitric acid before the addition of the phytate. The assay is standardised against a phosphate standard. 1 FTU of phytase produces 1µmol of PO_4^{3-} per minute under these standard conditions.

2. Scope: The assay is suited for determination of the phytase activity of animal feeds containing >50 FTU/kg.

3. List of Equipment:

i) pH meter with two-decimal digital readout, equipped with suitable electrodes. The pH meter is used to check and adjust the pH of the buffer and substrate solutions.

ii) Spectrophotometer providing three-decimal digital readout of absorbance (optical density, OD) at 415 nm, equipped with a 10mm flow cell.

iii) Thermostatted water bath capable of maintaining 37 ± 0.2°C.

iv) Centrifuge capable of providing about 2000 rcf (about 3000 rpm with a typical bench top centrifuge). The centrifuge is used : a) to separate the bulk of insoluble material from the feed extracts, in order to facilitate sampling, and b) to produce clear solutions for spectrophotometric measurement.

v) Centrifuge tubes of glass.

vi) Magnetic stirrers.

vii) Balances, capable of weighing the specified amounts to within ±1%.

viii) Vortex mixer.

ix) Volumetric equipment: A range of repeating pipettes, dispensers and volumetric glassware is required. The equipment used should deliver the specified volumes to within ±1%.

4. List of Chemicals:

Acetic acid, glacial (100%), p.a., Merck 63

Ammonia solution 25%, p.a., Merck 5432

Ammonium heptamolybdate tetrahydrate, p.a., Merck 1182

Ammonium monovanadate, p.a., Merck 1226

Calcium chloride dihydrate, p.a., Merck 2382
Nitric acid 65%, p.a., Merck 456
Potassium dihydrogen phosphate, p.a., Merck 4873
Sodium acetate trihydrate, p.a., Merck 6267
Sodium phytate, from rice, Sigma P-3168

5. List of Reagents:

5.1. Diluted nitric Acid: Dilute 1 volume of nitric acid (65%) with 2 volumes of water. Store at room temperature. Maximum storage time: indefinite.

5.2. Ammonium heptamolybdate reagent: Weigh out 100.0 g of $(NH_4)_6Mo_7O_{24}.4H_2O$. Dissolve in approximately 800 ml of water. Add 10 ml of 25% ammonia solution. Add water to final volume 1000 ml. Store at room temperature in the dark. Maximum storage time: 8 weeks.

5.3. Ammonium vanadate reagent: Weigh 2.35 g NH_4VO_3. Dissolve completely in 400 ml of water, preheated to 50-60°C. Then add 20 ml of diluted nitric acid (see 5.1, above). Add water to final volume 1000 ml. Store at room temperature in the dark. Maximum storage time: 8 weeks.

5.4. MoV stop reagent: Add 1 volume of ammonium vanadate reagent (see 5.3, above) to 1 volume of ammonium molybdate reagent (see 5.2, above). Then add 2 volumes of diluted nitric acid (see 5.1, above). Mix. Store at room temperature. Prepare fresh each day.

5.5. Acetate buffer, pH 5.5: Dissolve 150.1 g of sodium acetate trihydrate and 0.735 g of $CaCl_2.2H_2O$ in approximately 4500 ml of water. Adjust pH to 5.50 ± 0.02 with acetic acid (approximately 10 ml of glacial acetic acid). Add water to final volume 5000 ml. Store at room temperature. Maximum storage time: 1 week. Check and, if necessary, adjust pH on day of use.

5.6. 10% calcium chloride solution: Weigh out 100 g of

$CaCl_2.2H_2O$. Dissolve in water. Add water to final volume 1000 ml. Store at room temperature. Maximum storage time: indefinite.

5.7. Phytate substrate solution: Weigh out 1.40 g of sodium phytate. Dissolve in 200 ml of acetate buffer (see 5.5, above). Adjust pH at room temperature to 5.50 ± 0.02 by addition of diluted acetic acid (corresponding to approximately 0.4 ml of glacial acetic acid). Dilute with acetate buffer (see 5.5, above) to final volume 250 ml. Assuming that the sodium phytate is a decahydrate, with molecular weight of 1104, this substrate solution contains 5.1 mM phytate. Prepare fresh each day.

5.8. Phosphate stock standard solution (50 mM): Dry about 10 g of KH_2PO_4 in a vacuum oven at 105°C for 2 hours. Store in a dessicator. Weigh accurately 682 mg dried KH_2PO_4. Dissolve in 100 ml acetate buffer (see 5.5, above). Store at 0-5°C. Maximum storage time: 1 week.

5.9. 10 mM Phosphate standard: Dilute 10 ml of the phosphate stock standard solution (5.8) to 50 ml with acetate buffer (see 5.5, above). Prepare fresh each day.

6. Feed Sample: Grind a representative sample of the feed to a powder, for example using a household coffee grinder.

7. Method:

7.1. Feed Extraction: Weigh out 40.0 ± 0.25 g of the feed powder. Add 500 ml of deionised water containing 16 ml 10% calcium chloride solution (see 5.6, above). Stir vigorously for 60 minutes. Transfer approximately 5 ml into glass centrifuge tubes. Centrifuge, e.g. for 5 minutes at 3000 rpm. Prepare a series of glass test tubes. 4 tubes are needed for each feed sample (note that these tubes will be centrifuged after the enzyme reaction stage - their size and quality should be chosen accordingly). Pipette 100 µl of the centrifuged feed sample into each of the 4 tubes.

7.2. Analysis: Start the analysis sequence not later than 90 minutes after the start of the sample extraction procedure. 2 tubes are used for the enzyme reaction and 2 for blanks. Also prepare 4 tubes, each containing 100 µl of 10 mM phosphate standard (see 5.9, above), and 4 tubes containing 100 µl of acetate buffer (see 5.5, above). These are all treated in the same way as the enzyme sample tubes.

Enzyme samples, phosphate standard and buffer: 15 second intervals. At time zero (t = 0), add 3.0 ml phytate substrate (5.7), mix and place in the 37°C water bath. At t = 60 minutes, add 2.0 ml of stop reagent (see 5.4, above), mix and allow to stand at room temperature.

Enzyme blanks: As soon as all the samples have been started, prepare the blanks as follows: Add 2.0 ml stop reagent (see 5.4, above) followed immediately by 3.0 ml of phytate substrate (see 5.7, above); mix and allow to stand at room temperature. At t = 70-90 minutes, centrifuge all tubes for 10 minutes at 3000 rpm. At t = maximum 120 minutes, measure OD at 415 nm, using water as reference.

8. Calculation: For each sample and the phosphate standard, calculate mean values for the duplicates and calculate ΔOD_{415} by subtracting the blank values (the blank value for the phosphate standard is the assay value for the buffer sample).

Concentration of 10 mM phosphate standard solution: "P" mM (from weight of KH_2PO_4 used and the dilution).

mmol of PO_4^{3-} in phosphate standard assay: "P x 0.1"

ΔOD_{415} phosphate standard: "A".

Weight of sample in assay (from weighing, dilution and final

volume used): "E" g.

ΔOD_{415} of sample: "B".

Calculate sample phytase activity as µmol phosphate released per minute per g of sample. Sample phytase activity, FTU/g = (B x P x 0.1) / (A x E x 60).

8.1. Example: 40.2 g of feed extracted in 500 ml water plus 16 ml $CaCl_2$ solution. Feed concentration = 0.0779 g/ml or 0.00779 g/100 µl.

OD_{415} feed sample assay = 0.506
OD_{415} feed sample blank = 0.252
ΔOD_{415} feed sample = 0.254

Actual concentration of phosphate standard = 9.62 mM
Phosphate in 100ml = 0.962 µmol

OD_{415} phosphate standard = 0.563
OD_{415} buffer blank = 0.197
ΔOD_{415} phosphate standard = 0.366

Feed sample phytase activity =
(0.254 x 0.962) / (0.366 x 0.00779 x 60) = 1.428 FTU/g

9. Notes:

9.1. Vanadate toxicity: Vanadate (Vanadium in oxidation state +5) is highly toxic (LD_{50} p.o. in rats 18 mg/kg). The MoV stop reagent contains 0.6 mg/ml vanadate. The final assay solutions (which require the most handling, including centrifugation) contain 0.24 mg/ml vanadate (and ca. 2% HNO_3).

9.2 Calcium addition in feed assays: Calcium addition is included in the above method for feed samples. Calcium addition

decreases the production of free phosphate during the sample preparation steps, i.e. decreases both blank and enzyme OD values.

9.3 The phosphate standard: The phosphate response is linear up to OD_{415} of at least 1.5. The phosphate standard measurement is extremely reproducible, and use of only one phosphate concentration gives a sufficiently precise calibration. ΔOD_{415} for the 10mM phosphate standard is close to 0.37.

9.4 Maximum sample concentration: OD_{415} should not exceed 0.8. If higher values are obtained, adjustment of sample dilution is advisable. For example, use 1000 ml deionized water instead of 500 ml in 7.1 (*vide supra*).

Steam-heating

A portion of each phytase-enriched feed formulation, as well as a portion of the feed formulation to which no phytase had been added, was then subjected to a steam-heating procedure in which the conditions of the steam-heating procedure as used in a typical feed pelletizing process are simulated: 100 g of the feed formulation in question was transferred to a Büchner funnel (Schott Duran 21 341 44 05) and heated by passing steam up through the funnel and thus through the feed formulation contained in the funnel. Steam-heating was continued for 30 seconds, during which time the feed was stirred evenly. The supply of steam was kept constant throughout all runs and was sufficient to bring the temperature of the feed formulation up to >85°C at the end of the 30 second heating period. The heated feed formulation was then poured out of the funnel and cooled to ambient temperature.

The phytase activity of a sample of each of the steam-heated feed formulations was determined using Novo Nordisk analytical method ED-9513614 (*vide supra*), and the activity of each of the steam-heated, phytase-enriched preparations was corrected by

subtracting the residual, endogenous phytase activity determined for the steam-heated feed formulation to which no phytase preparation had been added.

The percentage retention of phytase activity in each of the steam-heated, phytase-enriched feed formulations was as given in the table below:

Phytase-containing granulate added to feed formulation	Retention of phytase activity (%)
PA	14
PB	14
PC	68
PD	56
PE	39

These results show that the use of granulate PC or granulate PD, both of which are preparations according to the present invention and have a high content of water-insoluble substances, including a hydrophobic substance, leads to markedly higher retention of enzymatic activity during the steam heating process than is observed for the granulates PA, PB and PE (the compositions of which are outside the scope of the present invention).

EXAMPLE 4

Effect of steam-heating on xylanase activity of xylanase-containing feed formulations: The degree of retention of enzymatic activity following steam-heating of feed formulations incorporating one of the xylanase-containing granulate preparations XA, XB, XC, XD and XE (see Example 2), respectively, was examined. A mash feed formulation having the same basic composition as that described in Example 3 (*vide supra*) was employed for this purpose.

For each of the xylanase-containing granulate preparations, the preparation in question was incorporated into the feed formulation so as to give a final xylanase activity in the range of about 800-1000 FXU/kg. In the case of granulates XA and XB, this was achieved by mixing an amount of granulate corresponding to 96000 FXU into 10 kg of feed formulation; the resulting portion of enzyme-enriched feed was then mixed thoroughly with 110 kg of the basic feed formulation, giving a total of 120 kg with a calculated activity of 800 FXU/kg. In the case of granulates XC, XD and XE, smaller batches (<1 kg) of enriched feed formulation were prepared.

The xylanase activity of a sample of each of the five resulting xylanase-enriched feed formulations was determined using Novo Nordisk analytical method ED-9511460.2, described below.

Analytical method

1. Analytical Principle: In Novo Nordisk analytical method ED 9511460.2, the xylanase is extracted from the feed sample into an aqueous solution. The enzyme is then allowed to react with a modified wheat xylan. This wheat xylan has been cross-linked (made insoluble) and dyed by coupling with a blue dye, and is available in tablet form. The reaction proceeds under standardised conditions (temperature 70°C, pH 6.0, reaction time 120 minutes) and is then stopped by increasing the pH. The solutions are filtered and the blue color of the solution, which is a measure of the enzyme-catalysed solubilisation of the substrate, is determined spectrophotometrically. Blank values are determined by carrying out the assay without addition of the enzyme sample. The assay is calibrated by adding a known enzyme activity to the feed sample. The increase in response due to the added enzyme activity is used to calculate the activity of the feed sample.

2. Scope: The assay is well suited for determination of the Bio-

Feed™ Wheat L xylanase activity of feeds that contain 50-250 FXU/kg. Higher activities can also be assayed, providing that the concentrations and dilutions are adjusted. Guidelines for the activity range 200-1000 FXU per kg are included herein.

3. List of Equipment:

i) pH meter with two-decimal digital readout, equipped with suitable electrodes. The pH meter is used to check and adjust the pH of the buffer solution.

ii) Spectrophotometer providing three-decimal digital readout of absorbance (optical density, OD) at 585 nm, equipped with a 10 mm flow cell.

iii) Laboratory benchtop centrifuge, used to separate the bulk of insoluble material from the feed extracts, in order to facilitate sampling.

iv) Centrifuge tubes for 5-10 ml samples.

v) Thermostatted water bath, capable of maintaining 70 ± 0.5°C.

vi) Vortex mixer.

vii) Magnetic stirrers.

viii) Glass-fiber filters, Whatman GF/C, 9 cm diameter.

ix) Balances, capable of weighing the specified amounts to within ±1%.

x) Volumetric equipment: A range of repeating pipettes, dispensers and volumetric glassware is required. The equipment used should deliver the specified volumes to within ±1%.

xi) Glass test-tubes, e.g. 15 mm x 100 mm, with caps.

4. List of Chemicals:

Disodium hydrogen phosphate dihydrate, p.a., e.g. Merck 6580

Sodium dihydrogen phosphate monohydrate, p.a., e.g. Merck 6346

Tris(hydroxymethyl)methane, e.g. Sigma 7-9, Sigma T-1378

Xylazyme AX, xylanase assay tablets, Megazyme Pty. Ltd., Australia

Bio-Feed™ Wheat L (xylanase preparation with known activity), Novo Nordisk A/S, Denmark.

5. List of Reagents:

5.1. Phosphate buffer, pH 6: Dissolve 60.0 g of $NaH_2PO_4.H_2O$ and 11.6g of $Na_2HPO_4.2H_2O$ in approximately 4500 ml of water. Adjust pH with 1N HCl or 1N NaOH to 6.00 ± 0.05. Dilute to final volume 5000 ml. Store at room temperature. Maximum storage time: 1 week.

5.2. Tris stop reagent: Dissolve 10.0 g of tris(hydroxymethyl)-methane in 1000 ml of water. Store at room temperature. Maximum storage time: 1 week.

5.3. Enzyme standard solution: From the Bio-Feed™ Wheat L (with known xylanase activity) prepare a solution in buffer with accurately known activity in the range 4.8-5.2 FXU/ml. For example, if the Bio-Feed™ Wheat L product has 500 FXU/g, weigh out accurately about 1000 mg and dissolve in 100 ml of phosphate buffer (see 5.1, above). Store at room temperature. Maximum storage time: 2 hours.

6. Feed Sample: The "feed sample" is the entire sample of feed that is received by the laboratory.

Sample for Bio-Feed™ Wheat xylanase Assay, FX: FX is a 100 g sample, taken from the "feed sample". The procedure that is used to take out FX must ensure that it is a representative sample. FX is ground to a powder which is then used in the assay. For repeat assays on the same "feed sample" the entire sampling procedure should be repeated.

7. Method: Weigh out 2 samples, each 40 ± 0.4 g, of the feed FX. Label as "a" and "b". In the following procedure treat both samples in the same way. At time zero (t = 0), add 800 ml of water and start stirring. At time t = 30 minutes, take out approximately 5ml and transfer to a centrifuge tube. Label as "aS" and "bS".

Add 1.0 ml of enzyme standard solution (see 5.3, above) and continue stirring. At time t = 45 minutes, take out approximately 5 ml and transfer to a centrifuge tube. Label as "aE" and "bE". Centrifuge all 4 tubes at approximately 3000 rpm for 5 minutes.

Prepare 16 glass test tubes, each containing 2.0 ml of phosphate buffer (see 5.1, above). Label consecutively from 1 to 16. These tubes are used as follows:
Tubes 1-3: assay, sample "aS".
Tubes 4: blank
Tubes 5-7: assay, sample "aE"
Tubes 8: blank
Tubes 9-11: assay, sample "bS"
Tubes 12: blank
Tubes 13-15: assay, sample "bE"
Tubes 16: blank

Transfer 200 µl of the feed extract centrifugates into the respective assay tubes. Mix with a vortex mixer. Add 200 µl of phosphate buffer (see 5.1, above) to the blank tubes.

Start the following assay procedure 60-90 minutes after starting the sample extraction: Using convenient time intervals between tubes, add 1 Xylazyme AX tablet to each of the 16 tubes, cap the tubes and place them in the 70°C water bath. Note that after addition of the tablet it is important NOT to mix in any way.

After 120 minutes in the water bath, the tubes are treated as follows: The tubes are taken up, 5 ml Tris stop reagent (see 5.2, above) is added, the cap is replaced and the contents are mixed well with a vortex mixer. Allow all tubes to stand at room temperature for 15 minutes, then mix again and filter through a Whatman GF/C filter into a clean tube. Measure OD at 585 nm for all 16 samples. Complete these measurements within 30 minutes after the filtration.

8. Calculation:
Calculate mean values of OD_{585} for "aS", "bS", "aE" and "bE" assays (mean of 3 measurements for each). Calculate the mean value of OD_{585} for the blanks. Calculate ΔOD for "aS", "bS", "aE" and "bE", by subtracting the mean blank value from the corresponding mean assay value.
Calculate (in FXU/kg feed) the enzyme activity added to the feed sample during the extraction (e.g. 1 ml with 5 FXU/ml added to 40 g feed gives 125 FXU/kg).
Calculate the activity in feed samples "a" and "b":

FXU/kg feed, sample "a" =
(ΔOD,aS) x (enzyme added, FXU/kg)/[(ΔOD,aE) - (ΔOD,aS)]

FXU/kg feed, sample "b" =
(ΔOD,bS) x (enzyme added, FXU/kg)/[(ΔOD,bE) - (ΔOD,bS)]

The xylanase activity of the "feed sample" is given as the mean of the "a" and "b" values.

9. Guidelines for Feed Samples with 200-1000 FXU/kg:
Increase the concentration of the enzyme standard solution (see 5.3, above) from 5 to 20 FXU/ml. Decrease the volume of feed extract centrifugate used in the assay from 200 µl to 50 µl. No other modifications are necessary.

Pelletizing/steam-heating

In the case of the xylanase-containing feed formulations prepared using the granulates XA and XB, respectively, the formulations were pelletized using a pilot scale steam conditioner (continuous horizontal mixer equipped with paddle blades and steam inlets). The flow rate through the conditioner was 200 kg/hour, the residence time in the conditioner was 30 seconds, and the steam input was adjusted to give a temperature

in the outlet feed stream of 95°C. From the conditioner outlet, the feed entered a Simon Heesen extruder and was pressed through a matrix with hole size 3mm x 35mm to form pellets. A sample of pellets was transferred to a cooler and was cooled to ambient temperature in a stream of air. A sample of the cooled pellets was taken for xylanase activity analysis.

In the case of the xylanase-containing feed formulations prepared using the granulates XC, XD and XE, respectively, a steam-heating procedure analogous to that described in Example 3 was employed. The supply of steam was kept constant throughout all runs and was sufficient to bring the temperature of the feed formulation up to >90°C at the end of the 30 second heating period. The heated feed formulation was then poured out of the funnel and cooled to ambient temperature.

The xylanase activity of a sample of each of the steam-heated feed formulations was determined using Novo Nordisk analytical method ED 9511460.2 (*vide supra*).

The percentage retention of xylanase activity in each of the steam-heated, xylanase-enriched feed formulations was as given in the table below:

Xylanase-containing granulate added to feed formulation	Retention of xylanase activity (%)
XA	35
XB	93
XC	10
XD	40
XE	85

These results show that the use of granulates XB and XE, which are both preparations according to the present invention and have a high content of water-insoluble substances, including a hydrophobic substance, leads to much higher retention of enzymatic activity during steam-heating in a pelletizing process or another steam-heating process than is observed for the granulates XA, XC and XD (the compositions of which are outside the scope of the present invention).

CLAIMS

1. A particulate, enzyme-containing preparation comprising: a total of at least 1% by weight (w/w) of one or more hydrophobic substances; and a total of at least 75% w/w of one or more water-insoluble substances, including said hydrophobic substance(s).

2. A preparation according to claim 1, comprising at least 5% w/w of said hydrophobic substance(s).

3. A preparation according to claim 1 or 2, comprising at least 90% w/w of said water-insoluble substance(s).

4. A preparation according to any one of claims 1-3, comprising 5-95% w/w of said hydrophobic substance(s) distributed substantially evenly throughout the material of the particles of said preparation.

5. A preparation according to any one of claims 1-3, comprising an enzyme-containing core, and a coating layer which contains said hydrophobic substance(s) and which surrounds said enzyme-containing core.

6. A preparation according to claim 5, comprising 1-50% w/w of said hydrophobic substance(s).

7. A preparation according to claim 5 or 6, comprising 5-15% w/w of said hydrophobic substance(s).

8. A preparation according to any one of the preceding claims, comprising, as a water-insoluble substance, a substance selected from the group consisting of inorganic salts and polysaccharides.

9. A preparation according to any one of claims 5-7, comprising, as a water-insoluble substance, a substance selected from the

group consisting of inorganic salts and polysaccharides.

10. A preparation according to claim 9, wherein said enzyme-containing core comprises cellulose in fibre form.

11. A preparation according to any one of claims 1-10, wherein said enzyme is an enzyme selected from the group consisting of phytases, phosphatases, xylanases, β-glucanases, α-galactosidases, α-amylases, β-amylases, cellulases, pectinases and peptidases.

12. A process for the production of an enzyme-containing animal feed composition, wherein an enzyme-containing preparation according to any one of claims 1-11, in admixture with other constituents of the final feed composition, is subjected to a steam-heating step.

13. A process according to claim 12, comprising a procedure whereby said final feed composition is pelletized.

14. Use of a particulate, enzyme-containing preparation according to any one of claims 1-11 in the production of an enzyme-containing animal feed composition.

15. An enzyme-containing animal feed composition produced using, as an enzyme-containing starting material, a preparation according to any one of claims 1-11.

16. An enzyme-containing animal feed composition obtainable by a process according to claim 12 or 13.